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Award Number: DAMD17-98-1-8351

TITLE: Gene Regulation by Retinoid Receptors in Human Mammary Epithelial Cells

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REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1999	Annual (25 Sep 98 - 24 Sep 99)

4. TITLE AND SUBTITLE
Gene Regulation by Retinoid Receptors in Human Mammary Epithelial Cells

DAMD17-98-1-8351

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Retinoids are important mediators of normal growth and differentiation of epithelial cells and may play an important role in the chemoprevention of breast cancer. While normal mammary epithelial cells express retinoic acid receptors (RAR) a majority of breast cancers have selectively lost the expression of specific RAR isoforms. In order to investigate the molecular mechanisms by which loss of retinoic acid receptor function might promote the malignant transformation of mammary epithelial cells, we have inhibited retinoic acid receptor function in normal human mammary epithelial cells (HMECs). We observe that suppression of retinoic acid receptor function in HMECs results in dysregulated growth and inhibits structural differentiation. These observations lead us to hypothesize that retinoids and retinoic acid receptors may be important in regulating mammary epithelial cell growth and differentiation, and therefore, loss of retinoic acid receptor function might promote breast cancer carcinogenesis. The aim of this proposal is to identify and characterize genes activated or suppressed by loss of retinoic acid receptor function in human mammary cells. It is our hope that these studies will facilitate the understanding of normal mechanisms controlling growth and differentiation of HMECs and provide insight into how these normal processes may be altered in breast cancer.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
10

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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YES — For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46. We are exempt because we are only using comercial cells with no identifiers

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IV INTRODUCTION

1. Title: Gene regulation by retinoid receptors in human mammary epithelial cells.

2. Principal Investigator: Victoria L. Seewaldt, M.D.

Co-investigator: Jas Lang, Ph.D.

3. Key words: mammary carcinogenesis, retinoids, prevention, molecular biology, nutrition

4. Introduction:

Retinoids are important mediators of normal growth and differentiation of epithelial cells and may play an important role in the chemoprevention of breast cancer. While normal mammary epithelial cells express retinoic acid receptors (RAR) a majority of breast cancers have selectively lost the expression of specific RAR isoforms. This has lead to the hypothesis that loss of retinoic acid receptor function might be an important event in mammary carcinogenesis. In order to investigate the molecular mechanisms by which loss of retinoic acid receptor function might promote the malignant transformation of mammary epithelial cells, we have utilized a dominant-negative approach to inhibit retinoic acid receptor function in normal human mammary epithelial cells (HMECs). We observe in our *in vitro* system that suppression of retinoic acid receptor function in HMECs results in dysregulated growth and inhibits structural differentiation. **These observations lead us to hypothesize that retinoids and retinoic acid receptors may be important in regulating mammary epithelial cell growth and differentiation, and therefore, loss of retinoic acid receptor function might promote breast cancer carcinogenesis. The aim of this proposal is to identify and characterize genes activated or suppressed by loss of retinoic acid receptor function in human mammary cells.** We propose to investigate the differential expression of genes in normal HMECs relative to HMECs with suppressed retinoic acid receptor function exhibiting *in vitro* loss of structural differentiation. It is our hope that these studies will facilitate the understanding of normal mechanisms controlling growth and differentiation of HMECs and provide insight into how these normal processes may be altered in breast cancer.

V BODY

In order to test the hypothesis that loss of retinoic acid receptor function might promote breast cancer carcinogenesis we suppressed normal retinoic acid receptor function in normal human mammary epithelial cells (HMECs) by retroviral-mediated expression of the dominant-negative retinoic acid receptor (DNRAR), LRARα403SN. This DNRAR is a truncated mutant RARα which acts as negative transcriptional regulator by simultaneously blocking all wild-type RAR isoforms (α, β, γ). The DNRAR dimerizes with retinoid receptors and binds retinoic acid response elements suggesting that the DNRAR acts by forming transcriptionally inactive heterodimers that compete for DNA binding with the natural retinoid receptor heterodimers. The advantage of this approach is that it is possible to disable the entire retinoic acid receptor pathway. We observed that inhibition of RAR function by a dominant-negative approach in HMECs results in dysregulated growth and inhibited the formation of a polarized ductal epithelium, suggesting that ATRA and RARs also play an important role in regulating proliferation and differentiation in mammary cells.

Technical Objectives:

Objective I: Isolation and characterization of retinoid-responsive genes activated or suppressed by retinoic acid receptors in normal human mammary epithelial cells. Formation of a polarized epithelial ductal structure is a characteristic of normal mammary cells and serves to distinguish normal from malignant mammary epithelial cells. We observe that inhibition of retinoic acid receptors in HMECs by a dominant-negative retinoic acid receptor (DNRAR) results in dysregulated growth and an inability of cells to form a normal polarized ductal epithelial structure when cultured in 3-dimensional extracellular matrix culture.

A) Isolation: We will employ the techniques of "differential display" and representational difference analysis of cDNA to isolate RA-regulated genes activated or inactivated by retinoic acid receptors in HMECs. RNA for differential analysis will be obtained from control HMECs (normal phenotype) and from HMECs whose normal retinoic acid receptor function is inhibited by a DNRAR (abnormal phenotype) grown in the presence of extracellular matrix.

B) Characterization: The function of candidate genes will be examined using sense and anti-sense sequences cloned into either retroviral or inducible expression vectors. Genes will be expressed in parental HMECs. Cells will be analyzed for growth characteristics and the ability to form a normal epithelial ductal structure in prepared extracellular matrix.

DATA:

Data for Objective IA: Isolation of genes regulated by retinoic acid receptor function in normal human mammary epithelial cells.

Preparation of RNA isolated from HMECs cultured in a prepared extracellular matrix:

HMECs with and without the DNRAR were plated on the nonadhesive substratum poly(2-hydroxyethyl methacrylate) (polyHEMA, Sigma). Poly-HEMA coated plates were prepared using an initial concentration of 0.25 mg/ml in 95% ethanol before drying. Approximately 1×10^7 HMEC controls or HMEC whose normal receptor function is inhibited by a DNRAR were be plated per T-75 flask. Prepared extracellular matrix (Matrigel ™) were diluted to 1% (vol/vol, approximately 200 μg of total protein per ml) in

standard medium was added to the culture as an overlay. Total mRNA was be isolated by the cesium chloride centrifugation method. Yield of total mRNA per 20 T-75 flasks was approximately 1.0 mg.

Identifying differences in mRNA expression by representational difference analysis of cDNA:

Poly(A+) mRNA was isolated from vector controls HMECs and HMECs whose retinoic acid receptor function is inhibited by a dominant-negative retinoic acid receptor (HMEC-DNRAR+). Approximately 200 μg total RNA was used to prepare poly(A+) mRNA using DYNAL beads (DYNAL) following the manufacture's instructions. The yield was approximately 10 μg mRNA per sample. Differentially expressed cDNA was identified by PCR-Select Differential cDNA subtraction kit (Clonetech). cDNA from HMEC-DNRAR+ cells was used as the tester and HMEC vector controls were used as the driver. A subtracted cDNA library was generated from each of the cDNA obtained from HMEC-DNRAR+ as well as HMEC controls. A secondary PCR amplification was performed using nested primers to reduce background PCR products and to enrich for differentially expressed sequences. The PCR products were directly sub-cloned into the pT-AdvTAge PCR cloning kit. To ensure the identification of low-abundance sequences, the subtracted library was hybridized with forward- and reverse-subtracted cDNA probes. The forward-subtracted probe constituted subtracted cDNA library generated with HMEC-DNRAR+ and reverse-subtracted probe consisted of the HMEC vector control as a tested. Clones representing mRNAs that hybridized with only the forward-subtracted probe were considered truly differentially expressed. Isolated genes have been sequenced and compared against the GenBank and EMBL sequence databases for homology with known genes. Novel genes, especially those displaying classic transcription factor motifs, have receive priority in further characterization. Northern analysis using radiolabelled probes from candidate sequences are currently being performed to confirm differential expression in parental HMEC-DNRAR+ cells (and non-expression in HMEC controls). Full length cDNAs have been isolated from a commercial HMEC library (Clontech or Stratagene) for two genes and are we are currently in the process of isolating the remainder.

Summary of results to date:

96	clones initially screened
8	punative differentially expressed sequences
4	sequences sequenced
2	genes isolated

Sequences identified to date

Clone	Identification	E value	Isolated
M17	Homo sapiens beta-subunit signal transduction gene	e-63	yes
P8	Homo sapiens protein tyrosine phosphatase	e-144	yes
M15	Homology with mus musculus proteinase-3	e-42	no
M23	Homo sapiens p58 receptor	5e-32	no

Next goals:

Methods for Objective IB: Characterization of genes activated or inactivated retinoic acid receptors in human mammary epithelial cells .

To determine if the isolated gene can inhibit structural differentiation of HMECs *in vitro* several strategies will be employed: 1) The cDNA will be cloned into the retroviral vector such as LXSN (containing a selectable neomycin resistance marker) and transduced into parental HMECs. This approach allows determination as to whether a candidate gene can inhibit structural differentiation of HMECs; 2) The LXSN retroviral vector will be used to express anti-sense cDNA in the same target cells by reversing the orientation of the cDNA inserts; 3) An expression vector with inducible promoters such as the tetracycline-regulated tTA/tetO (35) system may be used to achieve regulated expression in target cells. HMECs expressing the isolated gene or anti-sense will be grown in a prepared extracellular matrix as described above. Cells will then be assessed by light microscopy and by electron microscopy for the ability of the target gene to alter the formation of a acinus-like structure *in vitro* and to suppress growth inhibition by the presence of extracellular matrix.

VI SUMMARY:

We have utilized a unique *in vitro* system to isolate genes regulated by retinoic acid receptors in normal human mammary epithelial cells. We have utilized the technique of RDA to isolate differentially expressed genes in our model system. We have identified to date 8 potentially differentially expressed sequences, have identified 4 genes, and have isolated 2 genes. The two genes that have been isolated are of particular interest because they are signal transduction genes and are potentiallyWe are in the process of confirming that these identified sequences are differentially expressed in our model system. Two genes have been isolated. Our next goals are to:

1) Sequencing of the remaining 4 clones.
2) Finalize the differential expression of our 4 sequenced genes by PCR and Northern Analysis.
3) Isolate remaining genes.
4) Determine if our isolated genes can inhibit structural differentiation of HMECs *in vitro*.

Request for Change of Co-Investigator

Previously, the grant had been budgeted for Dr. Schickwann Tsai at Mount Sinai Hospital to be our Co-Investigator. Funds were budgeted as a subcontract because Dr. Tsai and myself were at different institutions. We have requested that Dr. Jas Lang at Ohio State University be our new Co-Investigator. For this reason, funds to our Co-Investigator are now to be paid as funds within Ohio State and not as a "Sub-Contract".

REPORT OF INVENTIONS AND SUBCONTRACTS

(Pursuant to "Patent Rights" Contract Clause) (See Instructions on Reverse Side.)

Form Approved
OMB No. 0704-0297
Expires Jun 30, 1992

1a. NAME OF CONTRACTOR / SUBCONTRACTOR	c. CONTRACT NUMBER	2a. NAME OF GOVERNMENT PRIME CONTRACTOR	c. CONTRACT NUMBER	3. TYPE OF REPORT (X one)
The Ohio State University Research Foundation	DAMD17-98-1-8351	US Army Medical Research Acquisition Activity	DAMD17-98-1-8351	X a. INTERIM ☐ b. FINAL
b. ADDRESS (Include ZIP Code)	d. AWARD DATE (YYMMDD)	b. ADDRESS (Include ZIP Code)	d. AWARD DATE (YYMMDD)	4. REPORTING PERIOD (YYMMDD)
1960 Kenny Rod Columbus, OH 43210-1063	980925	MCMR-AAA-B 820 Chandler St. Fort Detrick, MD 21702-5014	980925	a. FROM 980925 b. TO 990924

SECTION I – SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR / SUBCONTRACTOR (If "None," so state)

a. NAME(S) OF INVENTOR(S) (Last, First, MI)	b. TITLE OF INVENTION(S)	c. DISCLOSURE NO., PATENT APPLICATION SERIAL NO. OR PATENT NO.	d. ELECTION TO FILE PATENT APPLICATIONS				e. CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER	
			(1) United States		(2) Foreign			
			(a) Yes	(b) No	(a) Yes	(b) No	(1) Yes	(2) No
	NONE							

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR / SUBCONTRACTOR		g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED	
(1) (a) Name of Inventor (Last, First, MI)	(2) (a) Name of Inventor (Last, First, MI)	(1) Title of Invention	(2) Foreign Countries of Patent Application
(b) Name of Employer	(b) Name of Employer	NONE	
(c) Address of Employer (Include ZIP Code)	(c) Address of Employer (Include ZIP Code)		

SECTION II – SUBCONTRACTS (Containing a "Patent Rights" clause)

6. SUBCONTRACTS AWARDED BY CONTRACTOR / SUBCONTRACTOR (If "None," so state)

a. NAME OF SUBCONTRACTOR(S)	b. ADDRESS (Include ZIP Code)	c. SUBCONTRACT NO.(S)	d. DFAR "PATENT RIGHTS"		e. DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S)	f. SUBCONTRACT DATES (YYMMDD)	
			(1) Clause Number	(2) Date (YYMM)		(1) Award	(2) Estimated Completion
Mount Sinai School of Medicine	1 Gustave L. Levy Place New York, NY 10029	P.O. 822775			Research	980925	990924

SECTION III – CERTIFICATION

7. CERTIFICATION OF REPORT BY CONTRACTOR / SUBCONTRACTOR (Not required if Small Business or ☐ Non-Profit organization.) (X appropriate box)

a. NAME OF AUTHORIZED CONTRACTOR / SUBCONTRACTOR OFFICIAL (Last, First, MI)	c. I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.	
Marta L. Morris		
b. TITLE	d. SIGNATURE	e. DATE SIGNED
Interim Director Health Sciences Office	Marta L. Morris	990903

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